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Gonadal malignancy in 13 consecutive collected patients with disorders of sex development (DSD) from Semarang (Indonesia)

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ABSTRACT

Aims Caucasian patients with disorders of sex development (DSD) are at a high risk of developing germ cell cancer (GCC). GCC is prominent in young adults in Western countries, while the incidence is significantly lower in Asia. So far, the risk of GCC in Asian DSD patients is unknown.

Methods and results A detailed study of gonad histology , morphology and immunohistochemistry (OCT3/4, testis-specific protein Y-encoded, VASA, SCF/KITLG, SOX9, FOXL2) of 16 Indonesian DSD patients was undertaken. 13 cases could be analysed, including ovarian tissue (n=3), streak gonad (n=1), undifferentiated gonad (n=1) and testicular tissue (n=8), diagnosed as 46, XX (n=1), 46, XY (n=7) and sex chromosome DSD (n=5). The precursor lesion gonadoblastoma or carcinoma in situ, or GCC was diagnosed in four cases (30.8%; three 46, XY and one sex chromosome DSD). A hormone producing ovarian Leydig cell tumour was identified in a 46, XX patient, supposed to be a late onset congenital adrenal hyperplasia.

Conclusions In spite of the significantly lower risk of GCC in the general Asian population, DSD is a dominant risk factor. The study demonstrates the power of immunohistochemical markers for (early) diagnosis. This knowledge will deepen understanding of the pathobiology of GCC and clinical handling of patients with DSD, globally.

INTRODUCTION

Testicular germ cell cancer (GCC) is the most frequently found malignancy in Caucasian males aged 20–45 years and has increased over the last decades.[1 2] The highest incidence exists in Nordic countries (11.5 per 100 000 men, although also heterogeneously), while it accounts for the lowest rates (1–2 per100 000) among the black and Asian populations. The rates are notably lower in East Asia (0.7–1.6 per100 000) compared with West Asia (4.1 per100 000).[3] Among the known risk factors for GCC are a previous GCC, cryptorchidism, infertility (suggested to be part of the testicular dysgenesis syndrome) and disorder of sex development (DSD).[4 5] DSD patients are currently classified based on a three root system, in which the first step divides the patients into 46, XX DSD, 46, XY DSD and sex chromosome DSD.[6–8] Certain DSD subgroups are prone to develop GCC, in even up to 60% of the patients.[9] This risk is related to the presence of a specific region of the Y chromosome, referred to as gonadoblastoma locus on the Y chromosome (GBY), for which the testis-specific protein Y-encoded (TSPY) is the main candidate.[10 11] High risk is divided among gonadal dysgenesis (GD) patients who have the GBY region in their genome combined with intra-abdominal gonads (15–35%); non -scrotal gonads specifically in partial androgen insensitivity syndrome patients (50%). In termediate risk includes variants of Turner syndrome (Y+) (12%) as well as 17-β-hydroxysteroid dehydrogenase deficiency patients (28%). These forementioned DSD variants are all part of the 46, XY DSD entity.[7 8] At low risk of 2%, 3% and 1%, are patients with complete androgen insensitivity syndrome, ovotesticular DSD and Turner syndrome without Y-chromosomal material, respectively. Patients with GD and mosaic GD (45X/46, XY) show a prevalence of 30% and 15–40%, respectively, while the risk in cases of 5-α-reductase deficiency and Leydig cell hypoplasia is still unknown.[12]

Development of an invasive GCC is preceded by CIS (carcinoma in situ)/intratubular germ cell neoplasia, unclassified of the testis or gonadoblastoma (GB) of the dysgenetic gonad.[13] The formation of these precursors is dependent on the level of testicularisation of the gonad, directly related to the presence of SOX9 as marker for Sertoli cells and FOXL2 as marker for granulosa cells.[14] Long term effects on quality of life, including fatigue, metabolic syndrome, heart and vascular diseases, as well as secondary cancers, have been identified that are linked to application of irradiation and chemotherapy at a relatively young age.[15] Therefore, identification of the GCC as early as possible is of relevance for optimal treatment with long-term effect.[16]

Here we present results of the first ever series of histopathological characterisation of consecutively collected gonadal tissues of Indonesian DSD patients. Various germ cell and stromal markers, for the identification of GCC, were applied at the earliest possible pathogenetic stage.

MATERIAL AND METHODS

Patient selection and tissue samples

Indonesian DSD patients with ambiguous genitalia or any anatomical abnormality of external or internal genitalia, including penoscrotal hypospadias, with or without descended testes were examined at the Center for Biomedical Research, Diponegoro University/Dr Kariadi Hospital from

2004 to 2011 and diagnosed in accordance with the recent consensus on DSD.[7] Chromosome analysis was conducted using a G-banding technique at the Center for Biomedical Research, Diponegoro University, as described.[17] Hormonal and mutation analysis was performed in the laboratories at the departments of Endocrinology and Clinical Genetics (Erasmus MC), respectively. Supposed gonadal tissue samples of 16 patients with androgen action disorders (n=2), androgen excess (n=1) and GD (n=13), aged 0.9–30.9 years (median age at time of operation was: 19.9 years) were obtained after biopsy or gonadectomy. Bilateral specimens were available from five (31.2%), while the others were from unilateral biopsy or gonadectomy. Only 13 patients were included in the final examination. Characteristics of the patients are summarised in table 1. All gonadal samples were acquired for prophylactic and diagnostic purposes with informed written consent. Histological and immunohistochemical analyses (Octamer-binding transcription factor 4 (OCT3/4), Testis-specific Y-encoded protein 1 (TSPY), VASA, stem cell factor (SCF), SRY-related HMG-box, gene 9 (SOX9), Forkhead box L2 (FOXL2) and inhibin (INH) α subunit) were done at the departments of pathology at Diponegoro University, Indonesia and at Erasmus MC, Josephine Nefkens Institute, Rotterdam, The Netherlands.

Histological analysis

Specimens were first examined using routine H&E staining. Gonadal differentiation was determined per sample (testis/ovary/streak/undifferentiated gonad) as described previously.[18] General morphology and maturity of seminiferous tubules and germ cells, presence of supporting cells, for example, Sertoli cells, Leydig cells and granulosa cells, adnexal structures, for example, fallopian tube or uterus, were assayed. A Johnson score was attributed to each sample containing seminiferous tubules.

Diagnoses of the precursor lesions (CIS and GB) and their invasive GCC counterpart was made according to the WHO classification by an experienced pathologist (JWO). On the basis of morphology, germ cells were classified as immature or mature.[1]

RESULTS

General histology

Histological examination of the available gonadal samples demonstrated the following pattern: ovarian structures (n=3); testicular structures (n=8); streak gonad (n=1) and undifferentiated gonads (n=1) (table 2). Overall, the anatomical localisation of the gonads was 50% abdominal, 46.2% inguinal and 3.8% scrotal (one out of 26). However, within the group of 46, XY DSD the anatomical localisation was mainly inguinal (78.6%) (table 1). In case of the presence of testicular tissue (n=8), atrophic seminiferous tubules (based on Johnson score) were found in nearly all cases (n=7) (table 2). Maturation of spermatogenesis was variable, however elongated spermatids were never found. The Johnson score ranged from 1 to 6 with a mean of 2.5. Two patients with sex chromosome DSD had Johnson scores of 2 and 2.5, respectively.

The presence of germ cells was recognised by cytoplasmic staining for VASA and TSPY. In total, GCC was identified in four cases (30.7%), predominantly in the 46, XY DSD group (42.9%), and one in the sex chromosome DSD group (20%). All contained the Y chromosome in their karyotype, in agreement with the results of TSPY staining. The presence of malignant cells was visualised using OCT3/4 staining, showing nuclear localisation in all the cases. In the 46, XY DSD patients,

Table 1 Summary of clinical data and cytogenetic results for each patient

Case	Age*[19]	Gender at diagnosis time	Karyotype	Diagnosis	Genetic analysis	Phallus (cm)†	Right gonad Vol (ml)	Right gonad Location	Left gonad Vol (ml)	Left gonad Location	Quigley	Position of urethrae
46, XX DSD												
1	30.9	F	46, XX	Hypervirilisation e.c.Androgen producing tumour		2.5	NA	Abdominal	NA	Abdominal	4	Perineal
46, XY DSD												
2	13.8	F	46, XY	Gonadal dysgenesis	No SRY mutation	4.1	NA	Inguinal	NA	Inguinal	3	Perineal
3	23.3	F	46, XY	Gonadal dysgenesis	No SRY mutation	Normal clitoris	<1	Inguinal	<1	Inguinal	6/7	Perineal
4	1.9	M	46, XY	Androgen action disorder	No AR Mutation	3.5	1	Inguinal	NA	Abdominal	2	Scrotal
5	16	F	46, XY	Androgen action disorder	859delG	Normal clitoris	NA	Abdominal	NA	Abdominal	6/7	Perineal
6	1.8	M	46, XY	Gonadal dysgenesis	No SRY mutation	2.5	<1	Inguinal	NA	Inguinal	3	Scrotal
7	18.2	F	46, XY	Gonadal dysgenesis	DAX-1duplication	5.0	NA	Inguinal	NA	Inguinal	4	Perineal
8	27	F	46, XY	Gonadal dysgenesis	No SRY mutation	5.0	NA	Inguinal	1	Inguinal	3	Perineal
Chromosome DSD												
9	3.8	M	46, XY/46, XX	Gonadal dysgenesis	No SRY mutation	3.5	NA	Abdominal	1	Inguinal	2	Glans
10	22	F	46, XY/46, XX	Gonadal dysgenesis		Normal clitoris	NA	Abdominal	NA	Abdominal	6/7	Perineal
11	13	M	45, X/46, XY	Gonadal dysgenesis		4.0	NA	Abdominal	8	Scrotal	2	Scrotal
12	20.8	F	46X, idicY	Gonadal dysgenesis		6.0	NA	Abdominal	NA	Abdominal	3	Scrotal
13	19.8	F	45, X /46, XY	Gonadal dysgenesis		5.5	NA	Abdominal	NA	Abdominal	3	Perineal

*Age: Age when gonadectomised or biopsied.
†According to Judith G Hall, *et al*. http://www.amazon.com/Hanbook-Physical_Measurements-Medical-Publications/dp/019261696X-#Hand book of Normal Physical Measurements, Oxford University Press, 2003.
‡No gonadal histology.
DSD, disorders of sex development; e.c., et causa; NA, not available; SRY, Sex determining Region Y.

Table 2 Summary of patients' characteristics, histological finding and immunohistochemistry staining

Case	Age[19]	Side gonad	Location of gonad	Material from	Morphology (Johnson score)	OCT3/4	TSPY	VASA	SCF	SOX 9	FOXL2	Double staining	Others	Histology
46, XX DSD														
1	30.9	L	Abdominal	B	Ovary with gonadal stromal tumour compatible with a Leydig cell tumour lacking histological signs of malignancy. The histology does not reliably predict the clinical behaviour of the tumour (NP)	–	–	+ (in follicles)	NP	–	+	NP	Inh (+); LH-R (–)	Ovary with Leydig cell tumour
46, XY DSD														
2	13.8	L	Inguinal	G	Testicular tissue with many germ cells at different stages of maturation (6)	+	+	+	+	+	NP	OCT/VASA (+)	c-KIT (+); PLAP (+)	CIS
		R	Inguinal			–	–	–	–	+	NP	NP	NP	–
3	23.3	L	Inguinal	G	Testis with lLeydig cell hyperplasia. Most seminiferous tubules are atrophic (2–3)	–	+	+	–	+	NP	NP	NP	Leydig cell hyperplasia
		R	Inguinal			–	+	+	–	+	NP	NP	NP	Leydig cell hyperplasia
4	1.9	R	Inguinal	G	Dysgenetic gonad, no germ cells	–	–	–	–	+	–	NP	NP	Streak gonad, remnant sex cords, adjacent fallopian tube
5	16	L	Abdominal	G	Atrophic testis (1)	–	–	–	–	+	NP	NP	NP	Leydig cell hyperplasia
		R	Abdominal			–	–	–	–	+	NP	NP	NP	Leydig cell hyperplasia
6	1.8	L	Inguinal	G	UGT (NP)	+	+	+	+	+	+	OCT/TSPY (+)	NP	Gonadoblastoma
7	18.2	L	Inguinal	G	Testis with Leydig cell hyperplasia. Most seminiferous tubules are atrophic (1–2)	–	–	–	–	+	NP	NP	NP	Leydig cell hyperplasia
		R	Inguinal			–	–	–	–	+	NP	NP	NP	Leydig cell hyperplasia
8	27	L	Inguinal	G	Atrophic testes with seminoma and carcinoma in situ on both sides (1–2)	+	+	+	+	+	+	OCT/TSPY (+)	NP	CIS and seminoma
		R	Inguinal			+	+	+	+	+	+			CIS and seminoma
Chromosome DSD														
9	3.8	R (L in situ)	Abdominal	G	Ovary with multiple cysts, follicles and granulosa cells, including primordial follicles (NP)	–	–	+ (in cysts/ follicles)	NP	–	+	NP	c-KIT (–); PLAP (+)	Ovary with multiple cysts, follicles and granulose cells
10	22	R(L in situ)	Abdominal	G	normal ovary (NP)	–	–	+(follicles)	NP	–	+	NP	NP	Normal ovary
11	13	R(L in situ)	Abdominal	G	Atrophic testis, Johnson 1, Leydig cell hyperplasia, no CIS (2)	–	–	–	–	+	NP	NP	NP	Sertoli cells only
12	20.8	L(R in situ)	Abdominal	B	No germ cells; no malignancy; diffuse Leydig cell hyperplasia (2–3)	–	–	–	–	+	NP	NP	NP	Leydig cell hyperplasia
13	19.8	L(R in situ)	Abdominal	B	Testicular (NP)	+	+	NP	NP	NP	NP	NP	PLAP (+)	Seminoma

Age, Age when gonadectomised or biopsied; B, Biopsy; CIS, carcinoma in situ; DSD, Disorders of Sex Development; G, Gonadectomy; GCC, in situ and invasive; Inh, inhibin; L, left; LH-R, Luteinizing hormone Receptor; NA, not available: NP, not performed; PLAP, Plavental Alkaline Phosphatase; R, right; SCF, stem cell factor; TSPY, testis-specific protein Y-encoded; UGT, undifferentiated gonadal tissue.

all GCC containing gonads were located at an inguinal position, while in the sex chromosome DSD patient, the GCC affected an abdominal gonad. CIS was identified in two patients with high levels of Luteinizing hormone (LH) and Follicle Stimulating Hormone (FSH), low testosterone and no response for human Chorionic Gonadotropin (hCG) test. One presented unilaterally (case 2, figure 1), while the other was bilaterally adjacent to an invasive seminoma (case 8, figure 2), all at an inguinal position. Another pattern observed in a single patient (46, XY DSD) was the previously described undifferentiated gonadal tissue consisting gonadal tissue with germ cells without seminiferous tubule structures or follicles, mixed with Sertoli cells or granulosa cells in cord-like structures, supposed to be the precursor of GB (case 6, figure 3).[18] Indeed, a combined expression of SOX9 and FOXL2 in GB derived from undifferentiated gonadal tissue has been reported previously.[18]

No OCT3/4 positive germ cells were found in fully matured ovarian structures. SCF as a marker for early malignant germ cells was consistently expressed in invasive and precursor lesions.[19] OCT3/4 –TSPY or VASA coexpression was encountered in CIS as well as GB as seen in our double staining experiments (figure 4).

An unexpected Leydig cell tumour in a supposed late onset CAH patient

Within the series of patients investigated, a 46, XX virilised female presented with virilisation and secondary amenorrhoea. The testosterone value was tremendously increased (59.5 nmol/l, normal level 3.3 nmol/l) despite normal levels of LH and FSH (12.3 IU/l and 8.1 IU/l, respectively). Abdominal laparoscopy revealed a right normal ovary and a tumour lesion in the left, subsequently demonstrated to be a Leydig cell tumour confirmed by immunohistochemical detection of inhibin and LH receptor (data not shown). No mutation within exon 11 of the LH receptor was identified.[20]

DISCUSSION

We performed a detailed investigation based on histology and immunohistochemistry of a unique series of gonadal tissues from 13 DSD patients from the Indonesian population. To our knowledge, this represents the first of such a series and shows the high incidence of GCC and its precursor. Three malignancies were found in patients belonging to the category of 46, XY DSD and one case from sex chromosome DSD (46, XY/45, X). Indeed, these have been reported to show the highest risk for malignant transformation of germ cells leading to a GCC.[9 21 22] It is important to consider the presence of gonadal malignancy (ovarian Leydig cell tumour) in case of adult women with virilisation, which is a rare finding.[21]

Despite varying histopathological features, the general morphology of the DSD gonads and germ cells did not differ from that of the previous findings in Caucasian populations.[22 23] The majority of the gonads had a non-scrotal localisation. Most GCC or precursors were identified in gonads localised in the inguinal region and one in the abdominal region. This highlights the potent malignancy of partially descended testes, as compared with abdominal testes. In the latter position the germ cells are probably undergoing apoptotic cell death before they can give rise to malignancy.[23] With regards to age, 75% of GCC and their precursor lesions were at prepubertal and pubertal age ranges. The only two invasive GCC (seminomas) were found in two postpubertal patients (aged 19.8 and 27 years). This demonstrated the timing of progression from an in situ lesion to invasive growth happening after puberty.

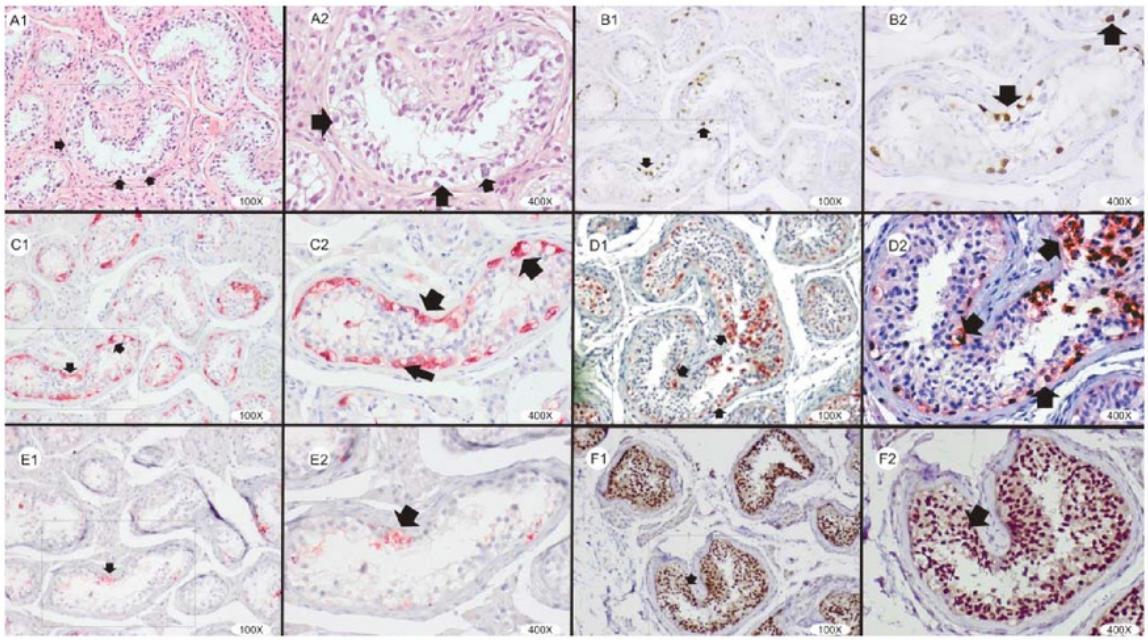

Figure 1 Case 2: 46, XY female with androgen action disorder. Breast development was present. After gonadectomy the patient received oestrogen hormonal therapy. H&E staining (A1, A2), morphology of the gonad showing testicular tissue with germ cells at different stages of maturation. Presence of carcinoma in situ (black arrow) confirmed by morphology and immunohistochemical staining for OCT3/4 (B1, B2), testis-specific protein Y-encoded (C1, C2), VASA (D1, D2), stem cell factor (E1, E2) and SOX9 (F1, F2). Magnifications are as indicated in each picture. This figure is only reproduced in colour in the online version.

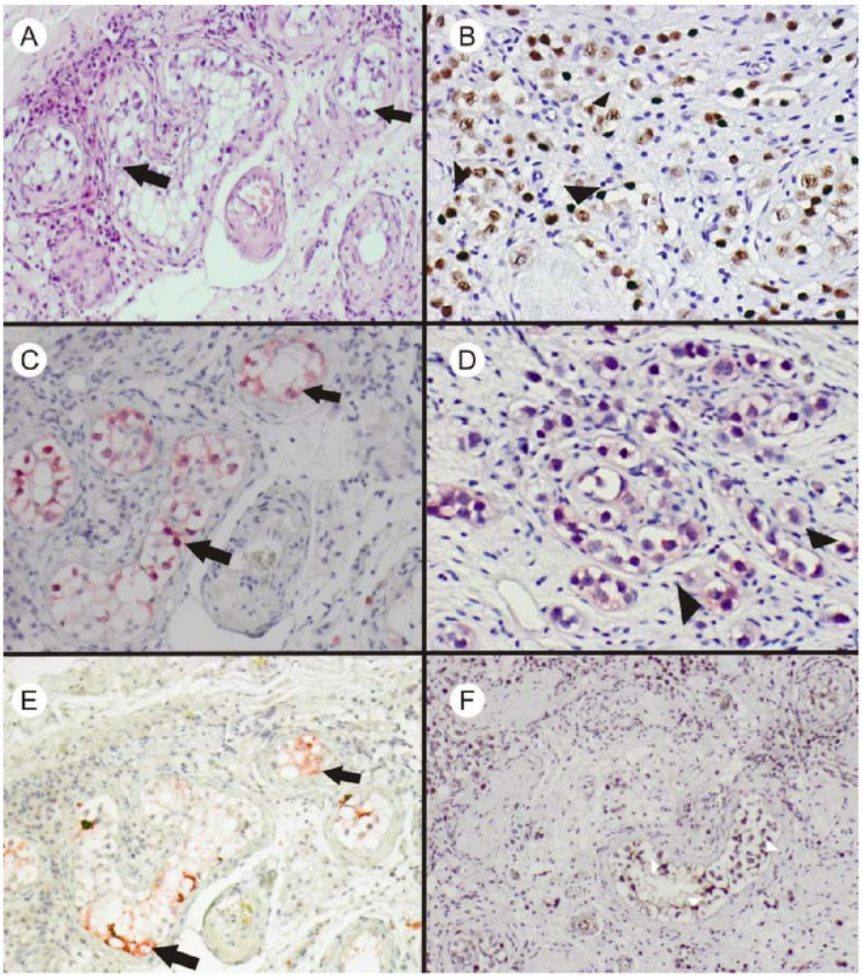

Figure 2 Case 8: 46, XY female with androgen action disorder. No breast development. After gonadectomy the patient received oestrogen hormonal therapy. Carcinoma in situ (CIS) (A,C,E) was found adjacent to seminoma (B,D). H&E staining (A) showing almost complete atrophy of the seminiferous tubules containing extensive CIS (black arrow), which also expressed testis-specific protein Y-encoded (C) and stem cell factor (E). Seminoma (black arrow head) found in the same sample expressed OCT3/4 (B) and VASA (D). Sertoli cells (white arrow head) identified by SOX9 (F). Magnifications are 200×. This figure is only reproduced in colour in the online version.

Infertility is a well-established feature of cryptorchid testis, also confirmed by this study based on the low Johnson score in inguinal and abdominal gonads, although increased proliferation of fetal gonocytes has been reported in case of testicular feminisation.[24 25] In general, germ cells express markers like TSPY and VASA [26 27], while OCT3/4 and SCF depend on the maturity of the germ cells, and their transformed phenotype. All GCC and the precursors, including dysgenetic and fetal gonads show the presence of OCT3/4, which is absent on fully matured (non-embryonic) germ cells.[11] OCT3/4 is reported to prevent apoptosis in embryonic germ cells, while in embryonic stem cells it mainly regulates pluripotency.[28] Again demonstrated in this study, CIS, seminoma and GB can be diagnosed based on OCT3/4 staining, even in the undervirilised gonad.[11] Similar to previous findings, abundant and abnormal expression of TSPY, which proposed to accelerate proliferation and promote tumourigenicity, was identified in all our cases.[11 25 29] However, TSPY can be disappeared upon progression to invasiveness.[11] This situation also applies to germ cells in an unfavourable environment in DSD situations as has been nicely illustrated in case 8 displaying a CIS adjacent to a seminoma. TSPY was abundantly expressed in the CIS component but strongly reduced in the seminoma as it gained its invasive capability. All gonads with high risk for malignant transformation also expressed SCF/kit-ligand (KITLG), confirming the use of this marker for detection of early malignant characteristics of GCC.[19]

Taken together, it is obvious that the use of specific immunohistochemical markers is highly informative for detection of GCC and its precursors; which is rarely applied in Indonesia. However, OCT3/4 staining alone, which can be done in Indonesia, is sufficient to detect early malignancy.

Incidence of GCC is low in most Asian countries like Indonesia.[2 3 30] However, within the group of DSD patients, the incidence is as high as found in the Caucasian DSD population, which is highly relevant.[1] This indicates that the combination of genetic and environmental factors, we refer to as 'GENVIRONMENT', which supposedly contribute to the high incidence of GCC in the Caucasian population, and which are

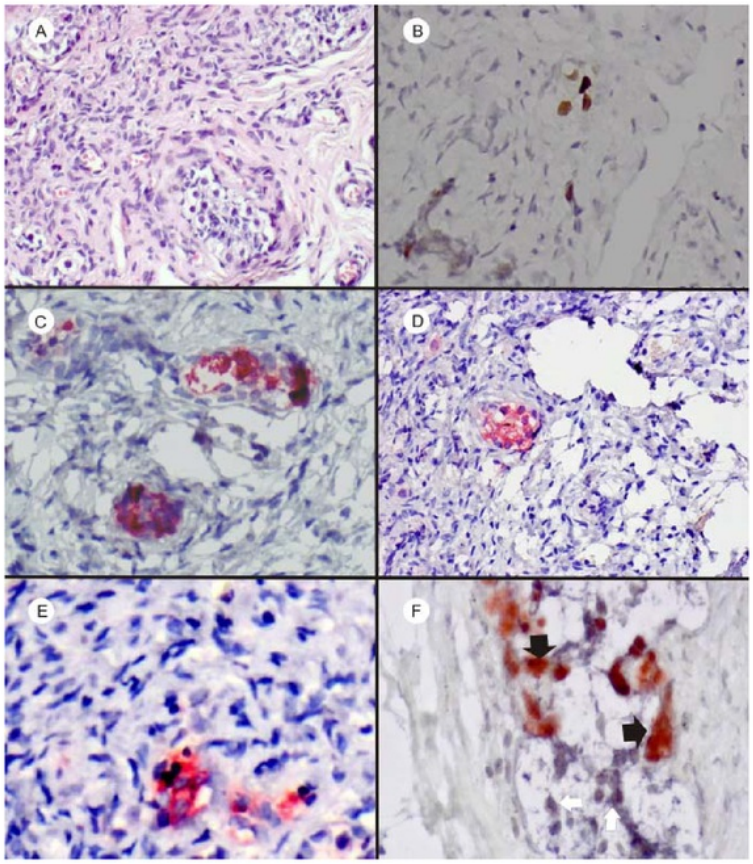

Figure 3 Case 6: 46, XY male with severe hypospadia and cryptorchidism staining depicting undifferentiated gonad with gonadoblastoma nest (black arrow) in H&E staining (A). Presence of immature germ cells confirmed by immunohistochemistry staining: OCT3/4 (B), testis-specific protein Y-encoded (C), VASA (D), stem cell factor (E). Supportive cells (F) are stained for SOX9 (white arrow) and FOXL2 (black arrow) indicative of Sertoli cells and granulose cells, respectively. Magnifications are 200×. This figure is only reproduced in colour in the online version.

less prominent in the Indonesian population, are compensated for in specific variants of DSD independent of ethnic background. Two recent studies indicate an association between specific genetic variants of the KITLG (as well as others) and the risk for GCC in the general population.[29 31] It is an intriguing hypothesis that the effects of environmental factors are likely modulated by genomic variation (polymorphisms), thus explaining the individual susceptibility and population-level differences in the incidence of GCC. It might be of interest to study the distribution of high risk alleles in the general, as well as in the Asian DSD population. Based on the first description of gonad histology of Indonesian DSD patients, it can be concluded that

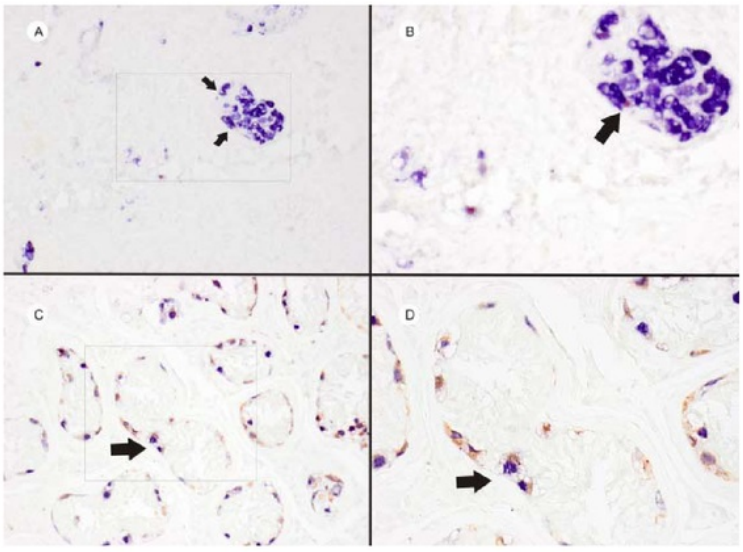

Figure 4 Representative examples of double staining experiments. Shown are testis-specific protein Y-encoded (blue cytoplasmic signal) and OCT3/4 (red nuclear signal) in case gonadoblastoma in undifferentiated gonad (case 6) (A, B); VASA (redcytoplasmic signal) and OCT3/4 (blue nuclear signal) in case of carcinoma in situ (case 2) (C, D). Note the double positive cells are indicated by a black arrow. Magnifications are 100× (left panel) and 400× (right panel). This figure is only reproduced in colour in the online version.

DSD is a dominant determinant for the development of GCC. This insight opens relevant new perspectives in the diagnosis and investigation of the pathogenesis of this type of cancer.

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Contributors AZJ and BAS contributed equally to the work. Other authors contributed significantly to the work by supporting laboratory, analysing material, and giving comments and suggestions for the paper.

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